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Note

Structural characterization of a water-soluble β-D-glucan from fruiting bodies of *Agaricus blazei* Murr

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Abstract

A β-D-glucan, Ab2-2N, was isolated from the hot-water extract of fruiting bodies of *Agaricus blazei* Murr by ethanol precipitation, anion-exchange and gel-permeation chromatography. Its structure was investigated by composition analysis, methylation analysis, Smith degradation, mild hydrolysis, and NMR spectroscopy. It contains a (1→6)-linked β-D-glucopyranosyl backbone, with one side chain consisting of terminal and 3-substituted β-D-glucopyranosyl residues attached at O-3 for every three backbone residues.

Keywords: β-D-Glucan; *Agaricus blazei*; Fruiting bodies; Mushroom

Agaricus blazei Murr is an edible mushroom distributed originally in Brazil and presently cultivated in other areas, including Japan, China and Indonesia. In Japan its fruiting body is generally called "Himematsutake" and is used as a health food and a home remedy. Its chemical components have been widely studied, including agaritine,[1] steroids,[2,3] lipids[4], lectin,[5] and various polysaccharides.[6,7] Its polysaccharide–protein complexes have been reported as an antitumor-active principle.[8,9] Mizuno recently reported another antitumor polysaccharide isolated from the mycelium of liquid cultivated *A. blazei* Mill, which has a (1→2)-linked β-D-mannan backbone and β-D-Glc-(1→3)-β-D-Glc-(1→ branches.[10] In this communication we report on one polysaccharide isolated from the fruiting bodies of *A. blazei*.

The crude polysaccharide Ab contained carbohydrate (57.5%) and protein (21.6%). Sugar analysis on Ab showed that the carbohydrate moiety was composed predominantly of Glc and small amounts of Rha, Xyl, Man and Gal in molar ratios of 74.6:4.5:3.7:9.7:7.5. After anion-exchange chromatography on a DEAE-Sephadex A-25 column and gel-permeation chromatography, the resulting Ab2-2N showed a symmetric peak on high-performance gel-permeation chromatography (HPGPC) (Fig. 1), indicating it was a homogeneous fraction. Ab2-2N is easily dissolved in water and DMSO, and contains 4.3% of protein and 89.8% neutral carbohydrate.

Sugar analysis showed that Ab2-2N contains only D-glucose. Although the *m*-hydroxydiphenyl method showed an uronic acid content of 5.5%,[11] TLC analysis of the hydrolyzate identified no uronic acid, indicating that the response in *m*-hydroxydiphenyl method probably resulted from a positive reaction from the neutral sugars and a contaminant. The average molecular weight was 1.7×10^5, estimated by HPGPC, in reference to dextrans of known molecular weights. The specific rotation is $-22.4°$ (*c* 0.26, water). The negative optical rotation indicates a β anomeric configuration for the glucosyl residues.

After three methylation procedures, IR indicated that the methylation was complete. The permethylated polysaccharide was subjected to acid hydrolysis, reduction and acetylation. The partially methylated alditol acetates were analyzed with GLC and GLC–MS, and the results are shown in Table 1. Methylation analysis

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indicated that Ab2-2N consists of (1→6)-linked, (1→3)-linked, (1→3),(1→6)-linked and terminal glucosyl residues. The high proportion of terminal glucosyl residues (19.3%) indicated that it was a highly branched polysaccharide. According to this result, three types of repeating units are possible for Ab2-2N: a (1→6)-linked backbone, a (1→3)-linked backbone, or an alternately (1→3),(1→6)-linked backbone. Therefore periodate oxidation and mild hydrolysis were performed for determination of additional structural features.

After periodate oxidation, $NaBH_4$ reduction, and dialysis, the remaining nondialyzable residue, Ab2-2NPA, was only a small proportion of the starting material, indicating that a substantial part of the polysaccharide had been degraded by oxidation. The result of methylation analysis for the nondialysable polyhydroxyl derivative is shown in Table 1. The presence of terminal and (1→6)-linked Glc indicates the incomplete oxidation of some residues. The Smith degradation product of this nondialyzate contained glycerol and an oligosaccharide as shown by TLC. This result excludes the possibility of a (1→3)-linked backbone. Methylation analysis revealed that the oligosaccharide contained terminal and (1→3)-linked Glc in the ratio of about 1:1, indicating that the (1→3)-linked Glc was attached to O-3 of the branching residues.

The anomeric configuration was revealed by the negative optical rotation and the absorption at 890 cm^{-1} in IR spectrum, and was further supported by the chemical shifts (~ 105 ppm) of the C-1 signal in the ^{13}C NMR spectra (Fig. 2). The signals were tentatively assigned in reference to the reported data,[6] and the results are shown in Table 2. Three closely located signals at 105.1 ppm, 104.7 ppm and 104.4 ppm in the anomeric region indicate that all the anomeric carbons adopt the β configuration. The strong signal at lower field (105.1 ppm) originated from (1→6)- and (1→3)-linked Glc due to their high amounts, and the other two from (1→3),(1→6)-linked and terminal residues, respectively. The substituted C-6 signal could be identified at 70.99 ppm from the reversed peak in the DEPT spectrum and the non-substituted C-6 signal at 62.91 ppm. The substituted C-3 signal appeared at 86.5 ppm, which is much weaker than the non-substituted C-3 signal at 78.07 ppm, indicates that most C-3 was not substituted.

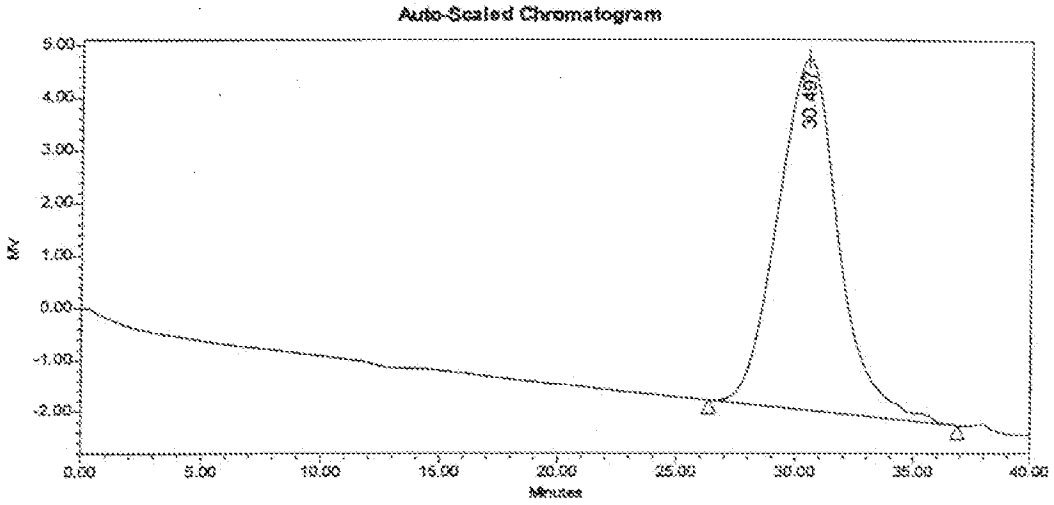


Fig. 1. High-performance gel-permeation chromatogram (HPGPC) of Ab2-2N.

Table 1
GLC–MS data of methylation analyses of Ab2-2N and Ab2-2NPA

Components	Molar ratios		Mass fragments (m/z)	Linkages
	Ab2-2N	Ab2-2NPA		
2,3,4,6-Me_4-Glc	19.3	0.36	45, 101, 117, 129, 145, 161, 205	Glc-(1→
2,4,6-Me_3-Glc	19.9	1.00	45, 101, 117, 129, 161, 233	→3)Glc-(1→
2,3,4-Me_3-Glc	41.3	0.57	99, 101, 117, 129, 161, 173, 189 233	→6)Glc-(1→
2,4-Me_2-Glc	19.1	0.77	117, 129, 139, 159, 189, 231, 233	→3,6)Glc-(1→

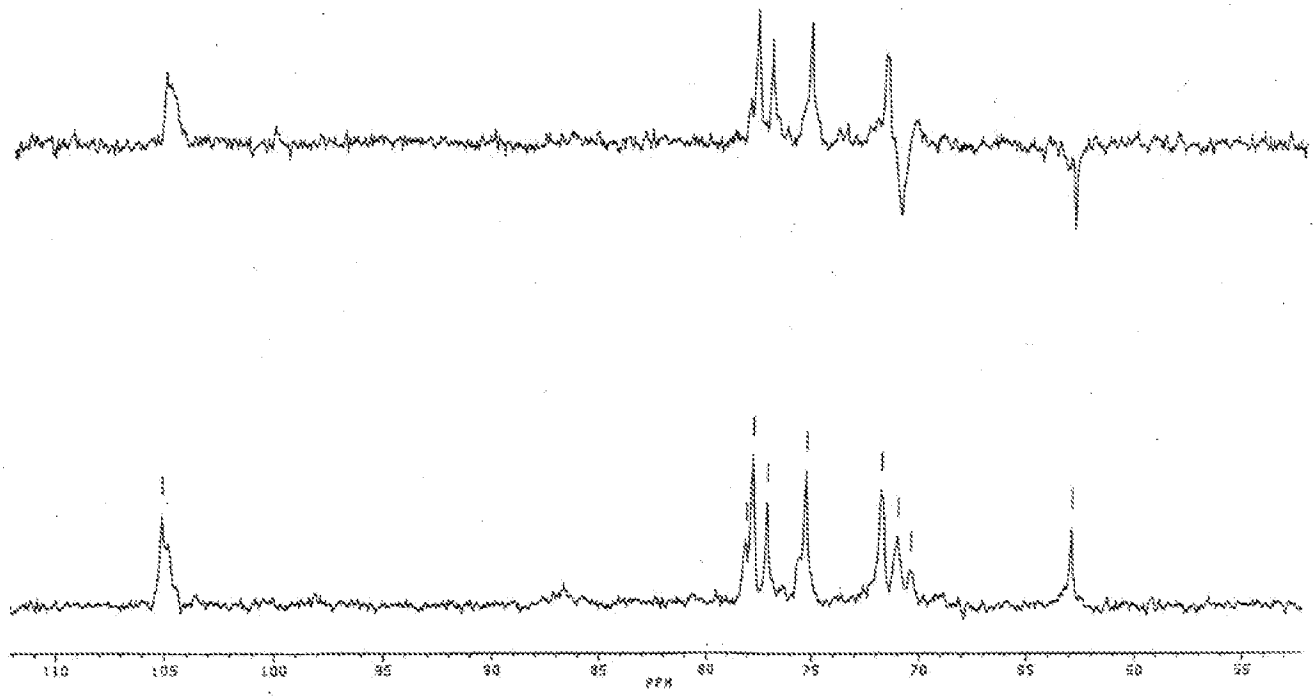


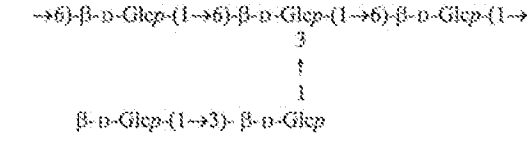
Fig. 2. ^{13}C NMR spectrum of native Ab2-2N.

Table 2
The ^{13}C NMR signals of Ab2-2N and their assignment

Chemical shift	Assignments	Chemical shift	Assignments
105.12	C-1 (1→3)- and (1→6-)-	77.06	C-5 (1→6)- and (1→3), (1→6)-
104.70	C-1 (1→3), (1→6-)	75.23	C-2
104.40	C-1 terminal	71.76	C-4
86.50	C-3 (1→3)- and (1→3), (1→6)-	70.99	C-6 (1→6)-
78.07	C-5 (1→3)- and terminal	70.39	C-6 (1→3), (1→6)-
77.75	C-3 (1→6)- and terminal	62.91	C-6 (1→3)- and terminal

On mild hydrolysis with 0.1 M TFA, 2N-PHP and 2N-PHS were obtained as the high-MW and low-MW fractions, respectively. In the ^{13}C spectrum of the degraded polysaccharide, 2N-PHP, the C-3 signal arising from (1→3)-linked Glc disappeared, indicating that 2N-PHP was a (1→6)-β-D-glucan, and approving that (1→3)-linked residues reside in side chains. This also excludes the possibility of a (1→6)-, (1→3)-alternatively linked backbone. The low-MW fraction was found to contain mainly an oligosaccharide by TLC, and the $NaBH_4$-reduced oligosaccharide 2N-PHS was subjected to methylation analysis. The result indicated it was an alditol of a disaccharide, 3-*o*-D-glucopyranosylglucitol. All these results support a (1→6)-D-glucan backbone with the branches attached at O-3, consisting mainly of a two-unit 3-*o*-D-glucopyranosyl-D-glucopyranosyl segment.

In summary, the results of methylation analysis, Smith degradation and partial hydrolysis, as well as the ^{13}C NMR data, indicate that Ab2-2N is a β-(1→6),(1→3)-glucan, containing a (1→6)-linked backbone, with one Glc-(1→3)-Glc-(1→ branch for every three backbone residues, attached at O-3. The average repeating unit was proposed for Ab2-2N as shown below. The side chains may not be so uniformly distributed along the backbone.



(1→6),(1→3)-Linked-β-D-glucans from *A. blazei* have been reported previously. Kawagish et al. had reported an antitumor β-(1→6)-glucan isolated from the same mushroom,[6] but it was isolated from 5% NaOH extract, water-insoluble, and did not contain (1→3) linkages. Mizuno et al. had reported a β-(1→6),(1→3)-glucan–protein complex,[7] but it exhibited a

positive specific rotation and was not structurally characterized in detail. In comparison, A2-2N is water-soluble, probably due to its highly branched structure, and showed a negative optical rotation. Preliminary in vitro trials showed that Ab2-2N exhibits a stimulating effect on ConA-induced T cell proliferation and LPS-induced B cell proliferation, especially at a concentration of around 10 μg/mL. The data are given in Table 3.

1. Experimental

Material.—The fruiting bodies of *A. blazei* were cultivated in Shanghai and obtained from Professor Qing-yao Yang, Department of Biology, Shanghai Teacher's University.

General methods.—The optical rotation was measured with a W22-1S polarimeter (Shanghai Physical Optics Instrument Co.), and the IR spectra were measured with a Perkin–Elmer 599B infrared spectrophotometer, either as a KBr pellet or Nujol as a mull. High-performance gel-permeation chromatography (HPGPC) was performed using a Waters 515 HPLC pump equipped with an Ultrahydrogel™ 500 and 2000 column (Waters Co.), monitored with a Waters 410 RI detector and 2487 UV detector (at 280 nm), using Dextran T-2000, T-500, T-110, T-70, and T-40 (Pharmacia Co.) as standards. GLCs were performed on a Shimidazu GC-14B chromatograph, equipped with a 3% OV-225 column (3.2 mm × 2.5 m) and an FID detector. The column temperature was kept at 210 °C for sugar analysis, and 190 °C for methylation analysis. GLC–MS was performed on a Varian VISTA 64 instrument, equipped with a SCOT DB-1701 column (25 m × 0.2556 mm, I.D.), with the temperature kept at 150 °C for 3 min, then raised to 250 °C at the rate of 2.5 °C/min.

Extraction and isolation.—The dried fruiting bodies of *A. blazei* were extracted twice with hot water (100 °C) for 4 h; the extract was concentrated by evaporation, and dialyzed against running water, and the nondialyzate was poured into three volumes of EtOH under vigorous stirring. This mixture was kept at 4 °C for 24 h, then centrifuged, and the precipitate was successively washed with EtOH and acetone, then dried in vacuo at 40 °C. The crude preparation thus obtained was a brownish powder designated as Ab.

Ab (2.8 g) was dissolved in water (50 mL), and the insoluble residue (0.38 g, 13.4%) was removed by centrifugation. The supernatant was applied to a DEAE-Sephadex A-25 column (Cl^-, 60 × 2.6 cm) and eluted first with water (400 mL), then with a 0–2 M gradient of aq NaCl (1 L). The carbohydrate was monitored with the phenol–H_2SO_4 method, and the protein with absorbance at 280 nm. After dialysis and lyophilization, Ab1 and Ab2 were obtained from the water and the NaCl eluates, respectively, in a yield of 336 mg (12.0% of Ab) and 695 mg (24.8%).

Ab2 was dissolved in distilled water (12 mL), and re-fractionated on a DEAE-Sephadex A-25 column (Cl^-, 60 × 2.5 cm), which was eluted stepwise with water (400 mL), 0.2 M NaCl (500 mL), 0.5 M NaCl (500 mL), and 1.0 M NaCl (500 mL), respectively. The polysaccharide fractions were recovered mainly in the 0.2 and 0.5 M NaCl eluate. After dialysis and lyophilization, Ab2-B2 (230 mg) was obtained from 0.2 M NaCl eluate, and Ab2-B5 (135 mg) from 0.5 M NaCl. Ab2-B2 was purified on tandemly linked Sephacryl S-300 (90 × 2.6 cm) and Sephadex G-200 columns (90 × 2.6 cm) with 0.2 M NaCl as solvent and eluent, with refractometric detection, giving Ab2-2N as the predominant fraction (140 mg). The homogeneity of Ab2-2N was estimated by HPGPC.[12]

Sugar analyses.—Ab2-2N was hydrolyzed in 2 M TFA at 110 °C for 1.5 h. After evaporation to remove TFA, one part of the hydrolyzate was analyzed with TLC on PEI-cellulose F plate (E. Merck), developed with 5:5:3:1 EtOAc–pyridine–water–AcOH, and visualized by spraying with aniline-*o*-phthalic acid reagent (1.6 g of *o*-phthalic acid dissolved in water–saturated *n*-butanol, containing 0.9 mL of aniline.) and heating at 105 °C for 5 min. The other part was transformed into the corresponding alditol acetates and analyzed by GLC as described previously.[13]

Protein, carbohydrate and uronic acid content determination.—Protein content was determined by the Lowry method with reference to bovine serum albumin,[14] and the carbohydrate determined by the phenol–H_2SO_4 method with Dextran T-300 (Pharmacia) as the reference.[15] Uronic acid content was estimated by the *m*-hydroxydiphenyl method.[11]

Table 3
Effect of Ab2-2N on proliferation of T- and B-cells by MTT assay [a]

Concentration (μg/ml)	Control	1	10	100
T cell	0.511 ± 0.032	0.588 ± 0.007	0.651 ± 0.014 [b]	0.553 ± 0.071
B cell	0.690 ± 0.015	0.742 ± 0.036	0.801 ± 0.033 [b]	0.798 ± 0.034

[a] Results are presented as mean ± SD ($n = 6$).
[b] $P < 0.05$, significant from the control.

Methylation analysis.—The polysaccharide, dried in vacuum at rt for 24 h, was methylated thrice according to the modified Ciucanu method.[16] The methylated polysaccharide was recovered by dialysis and freeze-drying. The completeness of methylation was confirmed by an IR spectrum (Nujol).

The permethylated polysaccharide was first depolymerized with 90% HCOOH at 100 °C for 4 h and then hydrolyzed with 2 M TFA at 100 °C for 5 h. The partially methylated sugars were reduced with $NaBH_4$, acetylated with acetyl anhydride, and then analyzed by GLC and GLC–MS.

Smith degradation.—Ab2-2N (25 mg), dissolved in 25 mL of 0.015 M $NaIO_4$ was kept at 5 °C in dark. The $NaIO_4$ consumption was monitored spectrometrically each day. After complete oxidation achieved (96 h), 0.2 mL of ethylene glycol was added to decompose the remaining $NaIO_4$. $NaBH_4$ (60 mg) was added and kept at rt for 6 h, with intermittent stirring. The mixture was neutralized to pH 7.0 with 1 M AcOH and dialyzed against distilled water (500 mL × 3). The nondialyzate was freeze-dried, designated as Ab2-2NPA (7.2 mg), one portion of which was analyzed by methylation analysis, and the remaining portion was hydrolyzed with 0.01 M TFA at rt for 48 h. After removing the TFA by evaporation, the hydrolyzate was analyzed by TLC, then passed through a Sephadex G-10 column, with refractometric monitoring. The carbohydrate fraction was subjected to methylation analysis.

Mild hydrolysis.—Ab2-2N (40 mg) dissolved in 0.1 M TFA (8 mL) was hydrolyzed at 100 °C for 1.0 h, and then dried by evaporation at < 40 °C. The residue was dissolved in water (4 mL), to which three vols of EtOH were added. The precipitate (2N-PHP) was washed with EtOH, dried in vacuo, and dissolved in D_2O, then the ^{13}C NMR spectrum was measured. The supernatant was dried by evaporation, analyzed by TLC, and the residue was dissolved in water (2 mL), and reduced with $NaBH_4$ (25 mg) at 25 °C for 2 h. After neutralization with 1 M AcOH, it was desalted using a Sephadex G-10 column (1.6 × 60 cm). The carbohydrate-containing eluate was pooled and freeze-dried to give 2N-PHS, which was subject to methylation analysis.

^{13}C NMR spectroscopy.—Ab2-2N (40 mg) was deuterium-exchanged and dissolved in 0.5 mL of D_2O and measured in a 5-mm tube with a Brucker AM 400 spectrometer at rt. All chemical shifts are referenced to Me_4Si.

Lymphocyte proliferation in vitro.—The mouse spleen cells were suspended to a final density of 5×10^9 cells/L in RPMI-1640 medium. Cells (100 μL/well) were added into a 96-well plate in the presence of the polysaccharide sample (1–100 mg/L) and ConA (5 mg/L) or LPS (25 mg/L). After incubation at 37 °C in a humidified 5% CO_2 incubator for 44 h, T- and B-lymphocyte proliferation was tested by MTT (5 g/L, 20 μL) assay.[17,18] The plate was incubated for another 4 h, and then the resolver (100 μL/well) was added. The absorbance was measured by DG-3022 ELISA at 570 nm.

References

1. Stijve, T.; Fumeaux, R.; Philipposian, G. *Dtsch. Lebensm-Rundsch.* **1986**, *82*, 243–248.
2. Kawagishi, H.; Katsumi, R.; Sazawa, T.; Mizuno, T.; Hagiwara, T.; Nagamura, T. *Phytochemisrty* **1988**, *27*, 2777–2779.
3. Takaku, T.; Kimura, Y.; Okuda, H. *J. Nutr.* **2001**, *131*, 1409–1413.
4. Ito, H.; Shimura, K.; Kawade, M.; Sumiya, T.; Yamada, K. *Igaku to Seibutsugaku* **1986**, *112*, 29–32; *Chem. Abstr.* **1987**, *106*, 149089a.
5. Kawagishi, H.; Nomura, A.; Yumen, T.; Mizuno, T.; Hagiwara, T.; Nakamura, T. *Carbohydr. Res.* **1988**, *183*, 150–154.
6. Kawagish, H.; Inagaki, R.; Kanao, T.; Mizuno, T.; Shimura, K.; Tto, H.; Hagiwara, T.; Nakamura, T. *Carbohydr. Res.* **1989**, *186*, 267–272.
7. Mizuno, T.; Hagiwara, T.; Nakamura, T.; Ito, H.; Shimura, K.; Sumiya, T. *Agric. Biol. Chem.* **1990**, *54*, 2889–2896.
8. Fujimiya, Y.; Suzuki, Y.; Oshima, K.; Kobori, H.; Moriguchi, K; Nakashima, H. *Cancer Immunol. Immunother.* **1998**, *46*, 147–159.
9. Fujimiya, Y.; Suzuki, Y.; Katakura, R.; Ebina, T. *Anticancer Res.* **1999**, *19*, 113–118.
10. Mizuno, M.; Minato, K.; Ito, H.; Kawade, M.; Terai, H.; Tsuchida, H. *Biochem. Mol. Biol. Int.* **1999**, *47*, 707–714.
11. Blumencrantz, N.; Asboe-Hansen, G. *Anal. Biochem.* **1973**, *54*, 484–489.
12. Wei, Y. A.; Fang, J. N. *Acta Pharm. Sin.* **1989**, *24*, 532–536.
13. Dong, Q.; Ding, S. W.; Yang, X.; Fang, J. N. *Phytochemistry* **1999**, *50*, 81–84.
14. Bensadon, A.; Weinstein, D. *Anal. Biochem.* **1976**, *70*, 241–250.
15. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
16. Needs, P. W.; Selvendran, R. R. *Carbohydr. Res.* **1993**, *245*, 1–10.
17. Xiang, D. B.; Li, X. Y. *Acta Pharmacol. Sinica* **1993**, *14*, 556–561.
18. Heek, K.; Reimann, J.; Kabelitz, D.; Hardt, C.; Wagner, H. J. *Immunol. Methods* **1985**, *77*, 237–246.